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Chapter 2

# Fibrinolysis at the Interface of Thrombosis and Inflammation — The Role of Neutrophil Extracellular Traps

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Additional information is available at the end of the chapter

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## 1. Introduction

As a response to inflammatory stimuli, polymorphonuclear (PMN, neutrophil) cells are able to expel a mixture of their nuclear and granular elements. These web-like substances are called neutrophil extracellular traps (NETs), structures that are able to entrap invading pathogens. NETs are composed of DNA, histones, granular enzymes and proteins (such as cathepsin G or elastase), and seem to be a universal tool of defense: humans, animals and even plants [1] are capable of extracellular trap formation, indicating that these webs provide an evolutionarily conserved protective mechanism.

Besides their protective function, a role for NETs is emerging in the pathogenesis of many diseases [2,3], and may be of interest regarding the pathogenesis of thrombosis. Stimulation of coagulation by NETs can result in unwanted thrombosis [4] and infection is a common event in the development of deep vein thrombosis [5,6]. Targeting the release of nucleosomes, development of NETs and the availability of circulating histones could be a strategy for prevention or therapeutic intervention in venous thromboembolism, sepsis and other diseases involving cell death and lysis.

This chapter describes the formation and structure of NETs and discusses the possible connections and interrelations between this newly recognized form of innate immunity and components of the haemostatic system.

## 2. Triggers of NET formation

NETs can be formed in response to all major types of microbes (bacteria, fungi, protozoa, viruses) and their products, as well as inflammatory mediators, ROS, cell-cell interactions, and certain non-infectious or non-physiological stimuli. Table 1. shows a set of examples for various triggers.

| Microbial stimuli | Chemical stimuli |
|---|---|
| **Bacteria** | **Microbial toxins and components** |
| *Enterococcus faecalis* | *δ-Toxin from Staphylococcus epidermidis* |
| *Escherichia coli* | *fMLP (+rapamycin)* |
| *Haemophilus influenzae* | *Glucose oxidase* |
| *Helicobacter pylori* | *M1 protein-fibrinogen complex* |
| *Klebsiella pneumoniae* | *Lipophosphoglycan* |
| *Lactococcus lactis* | *Lipopolysaccharide (LPS)* |
| *Listeria monocytogenes* | *Panton-Valentin leukocidin* |
| *Mannheimia haemolytica* | |
| *Mycobacterium tuberculosis/canettii* | **Inflammatory mediators and citokines** |
| *Serratia marcescens* | *Antibodies* |
| *Shigella flexneri* | *Calcium ions* |
| *Staphylococcus aureus* | *GM-CSF + C5a/ LPS* |
| *Streptococcus dysgalactiae/pneumoniae* | *Hydrogen peroxide* |
| *Yersinia enterocolitica* | *Interferon + eotaxin* |
| | *Interferon-α/γ + C5a* |
| **Fungi** | *Interleukin 1-β/8/23* |
| *Aspergillus fumigatus* | *Nitric oxide* |
| *Candida albicans* | *Platelet activating factor* |
| *Cryptococcus gattii/neoformans* | *Platelets through TLR-4* |
| | *TNF-α* |
| **Protozoa** | **Non-physiological stimuli** |
| *Leishmania amazonensis donovani/major/chagasi* | *Phorbol-12-myristate-13-acetate (PMA)* |
| | *PMA + ionomycin* |
| **Virus** | *Statins* |
| *Feline Leukemia Virus* | |
| *HIV-1* | |
| *Influenza A* | |

**Table 1.** Triggers of NET formation. Several microbial and chemical stimuli have been identified. A summary based on [7-10].

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## 3. Formation of NETs

### 3.1. NET formation as a form of cell death

NETs are the results of a unique cell death program that is different from apoptosis or necrosis [11]. It is characterized by the loss of intracellular membranes before the plasma membrane integrity is compromised (NETosis). To release NETs, activated neutrophils undergo dramatic morphological changes [12]. Minutes after activation by PMA, they flatten and firmly attach to the substratum, while showing a multitude of granules and a lobulated nucleus [13]. During the next hour, the nucleus loses its lobules, the chromatin decondenses and swells, and the inner and outer nuclear membranes progressively detach from each other. Concomitantly, the granules disintegrate. After one hour, the nuclear envelope seems to disaggregate into vesicles and the contents of nucleoplasm, cytoplasm and granules are able to freely mix. After approximately 4 hours, the cells round up and seem to contract until the cell membrane ruptures and the internal components are ejected to the extracellular space [13,14]. It is important to note, that depending on stimuli and donor, only a certain percentage of the activated neutrophils make NETs [13].

Apoptosis, another form of programmed cell death, is characterized by membrane blebbing, phosphatidylserine exposure on the cell surface, nuclear chromatin condensation and DNA fragmentation *without* membrane disintegration [8]. Necrosis is characterized by PS exposure during the early steps, cellular swelling and bursting, and plasma membrane damage/rupture *without* nuclear membrane disintegration. The program of NETosis, on the other hand, shows disintegration of the nuclear envelope *without* DNA fragmentation; loss of internal membranes and organelles, and membrane rupture (and therefore PS exposure) *after* mixing of the nuclear and cytoplasmic elements.

### 3.2. Alternative ways of extracellular trap formation

Besides the above described, first observed form of NETosis (also called *'suicidal' NETosis*), several other types have been reported [15].

In contrast with the PMA-induced 3-4 hour-long cell death program, a recently described form, *'vital' NETosis*, leads to rapid NET formation without neutrophil cell death [16-18]: Staphylococcus aureus appears to induce NETs in a rapid fashion [16], and LPS-activated platelets are also capable of inducing NETosis within minutes [19]. 'Vital' NETosis does not only spare the neutrophil from 'suicidal' lysis, but transforms them into anuclear cytoplasts capable of chasing and imprisoning live bacteria [18]. The third difference between 'suicidal' and 'vital' forms (besides timing and functional capacity of the involved neutrophils) is the mechanisms employed to create and cast out NETs: in contrast to the above described form, vital NETosis requires budding of the nuclear envelope, and vesicular trafficking of nuclear components to the plasma membrane, thereby delivering the NET out of the cell without requiring membrane perforation [16]. *Mitochondrial ETosis* originally observed in eosinophils, and later in neutrophils could also be considered as a subtype of the 'vital' form [20,21].

## 4. Structure and composition of NETs

NETs released from neutrophils in the extracellular space consist of nuclear DNA and various histones decorated with granular proteins. NETs are fragile, complex structures composed of smooth 'threads', approximately 15-25 nm in diameter, which are likely to represent a chain of nucleosomes from unfolded chromatin. High-resolution scanning electron microscopy (SEM) revealed that the NET threads are studded to variable extent with globuli of 30-50 nm [14] that contain the multiple cathelicidin antimicrobial peptides which originate from the neutrophil granules (or lysosomes). Several 'threads' can be wound into 'cables' that can be up to 100 nm in diameter (Figure 1.).

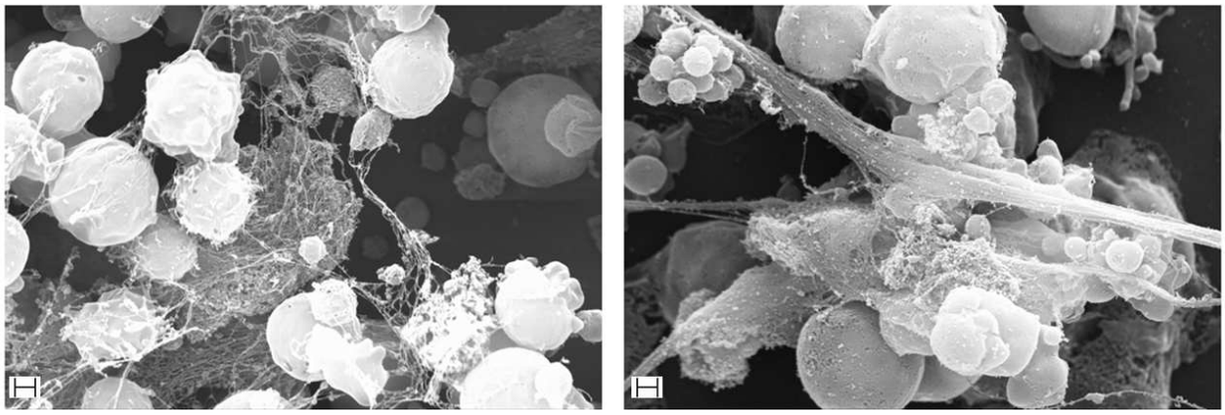

**Figure 1.** SEM images of NETs produced by PMA-activated neutrophils. Images are taken at 10,000x magnification. Scale bars=1 μm.

These cables then form complex three-dimensional structures that, using SEM, can be hard to distinguish from fibrin networks [22]. Analysis of cross sections of NETs by transmission electron microscopy (TEM) revealed that fibers are not surrounded by membranes [23]. When produced in multiwell plates in vitro, NETs float within the medium, rather like a spider's web does in moving air [24]. The fact that they are 'sticky' as a result of their electrostatic charge and that they extend over areas of several microns makes them very effective at trapping [25], and possibly killing microorganisms [24].

DNA is a major structural component, because several intercalating dyes stain NETs strongly, and deoxyribonuclease (DNAse) treatment results in the disintegration of NETs, whereas protease treatment has no such effect [23]. Accounting for approximately 70%, the most abundant component of NETs are histones [26]. All core histones as well as linker histones can be found in NETs (H1, H2A, H2B, H3, H4), although in an enzymatically processed form (see later). The aforementioned globuli contain proteins and enzymes from the primary (azurophilic) granules (e.g. neutrophil elastase, cathepsin G, myeloperoxidase, bactericidal permeability increasing protein BPI), secondary (specific) granules (e.g. lactoferrin), and tertiary granules (e.g. gelatinase or MMP-9, peptidoglycan recognition proteins PGRPs [27]) of

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neutrophils [28]. Cytoplasmic components, like calprotectin, a heterodimer of cytosolic S100A8 and S100A9, are rarely found in NETs [26].

These proteins exert various antimicrobial actions [29]: MPO is responsible for microbicidal HOCl generation; serine proteases (neutrophil elastase NE, cathepsin G, proteinase 3, tryptase, neutrophil serine protease 4 NSP4 [30]) are able to inactivate bacteria by cleaving their virulence factors [23]; cathelicidin LL37, BPI, defensins, and histones can disintegrate pathogen cell membranes challenging their viability [31,32]; calprotectin [26,33], calgranulin and lactoferrin chelate ions that are vital for microbial growth, altogether making NETs an effective tool virtually against all types of microbes.

NETs produced from mitochondrial DNA release have a slightly different structure [21]. NE and MPO co-localize with mitochondrial DNA, but certain nuclear (lamin B, nuclear matrix protein-45, poly-ADP-ribose polymerase, histones) and other (cytoplasmic caspase-3, beta-actin, mitochondrial cytochrome c, membrane markers CD15 and 16) elements are absent, which suggests a different type of host-NET interaction in the case of mitochondrion-derived NETs.

## 5. Intracellular events leading to NET formation

A unifying theory describing the subsequent steps of NET formation is still missing, but many mechanisms have been identified to contribute to NET expulsion.

### 5.1. Signaling events

The signaling mechanisms leading to the formation of NETs are poorly understood, and it is very likely that different triggers are able to induce NETosis through different pathways (Figure 2. [34]).

The protein kinase C (*PKC*) enzyme family is comprised of conventional, novel and atypical isoforms [35]. There are at least four conventional isoenzymes: PKC$\alpha$, PKC$\beta$I, PKC$\beta$II and PKC$\gamma$. The novel isoenzyme group has four subtypes: PKC$\delta$, PKC$\varepsilon$, PKC$\eta$ and PKC$\theta$. The third group, atypical isoenzymes, consists of PKC$\zeta$ and PKC$\iota$ [35]. PMA (phorbol-12-myristate-13-acetate), a widely used inducer of NETs, stimulates conventional ($\alpha$, $\beta$I, $\beta$II, $\gamma$) and novel ($\delta$, $\varepsilon$, $\eta$, $\theta$) PKC by mimicking the activating ligand diacylglycerol (DAG) [35]. PKC isoforms of all classes have been reported in neutrophils from healthy donors [36], and activation of PKC is critical in the generation of NETs [37]. Nevertheless, an intricate antagonism is present between PKC isoforms in the regulation of a crucial element of NETosis, histone deimination: PKC$\alpha$ has a dominant role in the repression of histone deimination, whereas PKC$\zeta$ is essential in the activation of peptydil arginine deiminase 4 (PAD4, see later) and the execution of NETosis. The precise balance between opposing PKC isoforms in the regulation of NETosis affirms the idea that NET release underlies specific and vitally important evolutionary selection pressures [38].

PK-C activation (e.g. by PMA) is upstream of the *Raf-MEK-ERK* pathway [39] leading to phosphorylation of gp91phox [40] and p47phox [41] which initiates the assembly of the cellular or phagosomal membrane-bound and the cytosolic subunits of another key player of NET formation, NADPH oxidase (see below). An alternative route for activation of ERK is also suggested through generation of reactive oxygen species (ROS) [42]. The Raf-MEK-ERK pathway also upregulates the expression of antiapoptotic protein Mcl-1, which contributes to the inhibition of apoptosis and redirects the death program to NETosis [39].

The monomeric G-protein (rho small GTPase) *Rac2* is also activated upstream of NADPH oxidase activation [43].

The role of *PI3K-Akt-mTOR* pathway is contradictory. Inhibition of mTOR leads to enhancement of fMLP-induced NETosis, because the pathway inhibits autophagy, a process that seems to enhance NET formation (e.g. by blocking apoptosis) [10]. If a different trigger, lipopolysaccharide (LPS) is used, however, mTOR seems to support NETosis by exerting translational enhancement of HIF1$\alpha$ [44].

Certain triggers of NETosis act through a PKC/ROS-independent pathway, possibly mediated by *Src* kinase [45], which may be able to directly activate PAD4.

Cytoskeletal elements may also play a role in transmitting signals from the cell surface to the nucleus, e.g. inhibition of the cell surface receptor integrin *Mac1-cytohesin1* (a guanine exchange factor)-*actin* cytoskeleton pathway results in inhibition of PAD4 activation and NET formation [46].

### 5.2. NADPH oxidase and ROS formation

Most pathways converge to activate *NADPH oxidase* as a key enzyme of the process [47]. Neutrophils isolated from patients with chronic granulomatous disease (CGD) caused by mutations in NADPH oxidase fail to produce NETs upon PMA-stimulation [13]. Inhibition of the oxidase with diphenyleneiodonium DPI also prevents NETosis in response to several factors [48]. Assembly of the NADPH oxidase responsible for the generation of ROS during the respiratory burst requires phosphorylation of the four cytosolic subunits (p47-phox, p40-phox, p67-phox and Rac) to enable their association with the membrane bound gp91phox-p22phox (cytochrome b558) complex. Once being in the active form, the enzyme generates ROS, out of which the most important seem to be the superoxide ions ($O_2^-$). $O_2^-$ dismutates (either spontaneously, or by superoxide dismutase [SOD] catalysis) to form $H_2O_2$. Further metabolization of $H_2O_2$ can lead to a variety of toxic oxygen derivatives, like the primary mediator of oxidative killing in the phagosome, HOCl, formed by *myeloperoxidase* (MPO) action. The importance of the latter enzyme is underlined by studies in patients suffering from MPO deficiency: the level of NETs they produced correlated with the degree of the enzyme deficiency [49]. How ROS generated during an oxidative burst contribute to NETosis is controversial. One possibility is that they contribute directly to the observed morphological changes by causing direct membrane destruction [50]. A proposed alternative is that ROS directly and indirectly (through activation of NF-κB) inactivate caspases [51-54], while exerting a possible autophagy-enhancing effect [34]. Both mechanisms lead to an inhibition of apopto-

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sis, ensuring that the already ongoing cell death program does not take an apoptotic route. ROS also play a crucial role in initializing the events that lead to chromatin decondensation, another key component of this type of cell death (Figure 2.).

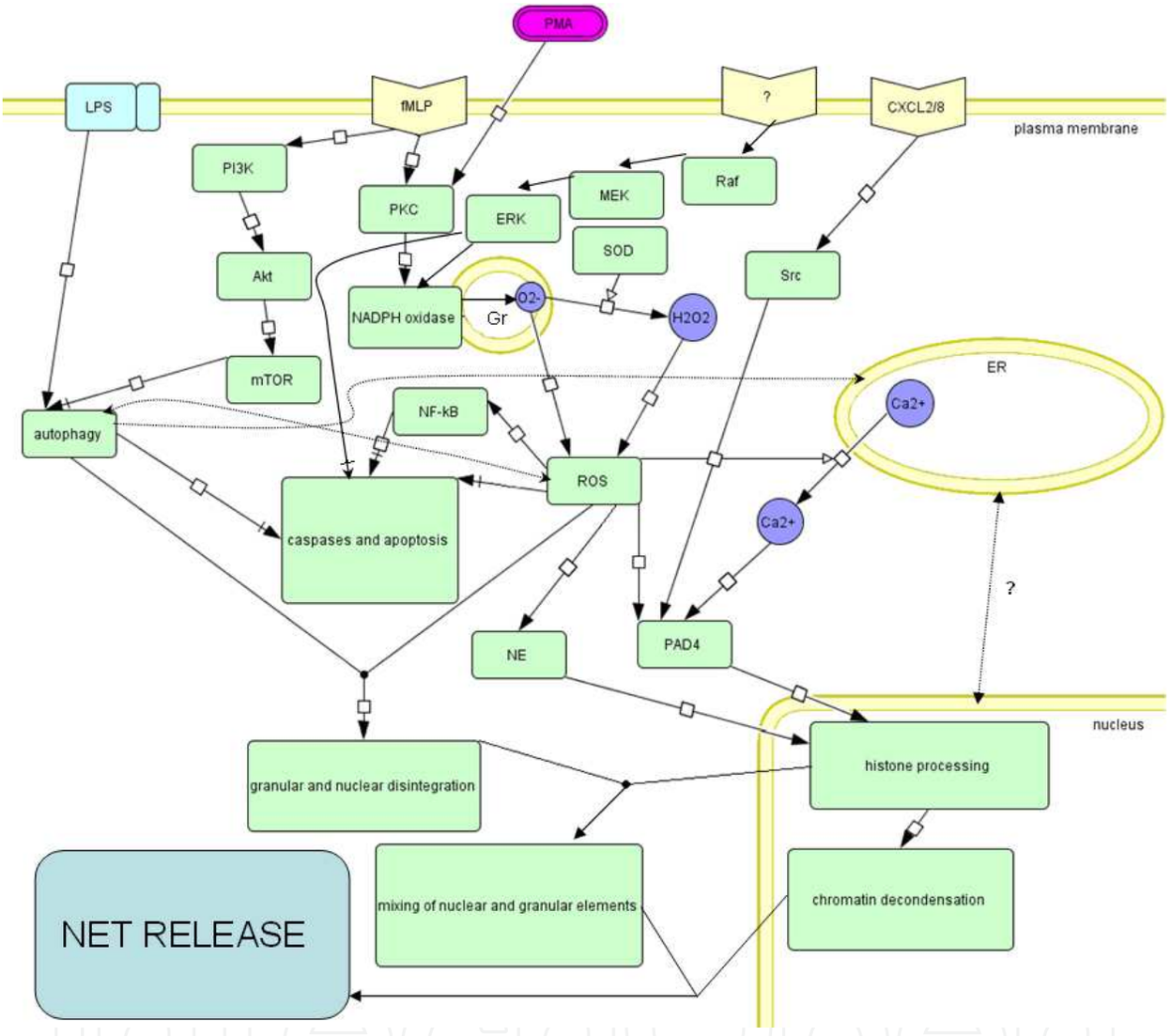


**Figure 2.** Intracellular steps leading to NET formation. Several signaling pathways can lead to NADPH oxidase activation and ROS formation, which triggers NE and PAD4 action on nuclear histones. Nuclear disintegration and decondensation leads to mixing of the granular and nuclear components, which are later expelled from the cell in the form of NETs. Dashed-end arrows represent inhibition, arrows pointing to the middle of another arrow represent activation of a step. Arrows with dotted lines stand for ambiguous relations. Gr: granule. For other abbreviations and explanation: see text. Modified from [34].

### 5.3. Chromatin decondensation

One option to weaken the interaction between DNA and highly positively charged histones is the enzymatic processing. At this moment, two enzymes seem to be of greatest importance: PAD4 (peptydilarginine deiminase 4) and NE (neutrophil elastase).

*Peptydilarginine deiminases* are enzymes catalyzing citrullination (deimination), a posttranslational modification of arginine to citrulline. The process results in the loss of positive charge and hydrogen bond acceptors, therefore leading to weakened protein-protein, RNA-protein, and DNA-protein interactions. Out of the five PAD enzymes expressed in humans and mice (PAD1-4 and 6) [55], PAD2 and 4 are the most abundant in neutrophil granulocytes, and the latter seems to be critical in NET formation: PAD4-deficient mice are unable to decondense chromatin or form NETs [56], whereas overexpression of PAD4 is sufficient to drive chromatin decondensation to form NET-like structures in cells that normally do not form NETs [57].

PAD4, a 74 kDa protein that exists as a head-to-tail dimer [58,59] is the only member of the peptydilarginine deiminase family containing a nuclear localization signal that ensures its trafficking to the nucleus [58,60,61] (although not the only one to be found inside, e.g. PAD2 is also reported to be localized intranuclearly [62]). The activation of PAD4 is calcium-dependent: binding of calcium to the C-terminal catalytic domain induces conformational changes that lead to the adequate positioning of critical active site residues [58]. The calcium-dependency of the enzyme also serves as a possible connection between ROS generation (possibly leading to calcium release from the endoplasmic reticulum) and PAD4 activation. In addition, ROS are possible direct regulators of PAD4 [63]. Cytoskeletal activity and autophagy may also be involved in PAD4 activation, since both processes have been shown to be required for chromatin decondensation during NET generation.

The main nuclear substrates of PAD4 are arginyl residues of PRMT1 (protein arginine methyltransferase 1) [61], PAD4 itself (autocitrullination downregulating the activity of the enzyme [64,65]), and, most importantly regarding the process of NETosis, histones (H2A, H3Arg-8 and-17 or H4Arg3) [66]. Hypercitrullination of arginil residues in histones [67] weakens their interactions with DNA resulting in the dissociation of heterochromatin protein 1-β [57], and the extensive chromatin decondensation that leads to nuclear delobulation and swelling of the nuclear content [66,68].

In concert with PAD4, *neutrophil elastase* (NE), a serine protease that is able to cleave histones, also promotes nuclear decondensation. H1 is cleaved early during the process of NETosis, but nuclear decondensation coincides with degradation of H4 [50]. ROS may play a possible role in the translocation of NE from the azurophilic granules into the nucleus by disrupting the association of NE with the proteoglycan (e.g. serglycin) matrix that is thought to down-regulate protease activity in resting cells [69-71]. The similar, but later occurring translocation route of myeloperoxidase MPO supports the process, which seems to be independent of its enzymatic activity [50]. Once in the nucleus, NE activity is reduced by DNA, which could help in protecting certain NET-components from losing their antimicrobial activity by proteolytic digestion [50]. Interestingly, serpinb1, an inhibitor of neutrophil proteases is also being transported to the nucleus during NETosis, possibly setting a brake of NE action [72]. While NE knockout mice fail to form NETs in a pulmonary model of Klebsiella Pneumoniae infection [50], serpinb1-deficient neutrophils produce overt NETosis in vivo during Pseudomonas aeruginosa lung infection [72], which points to the importance of the fine regulation of NE activity during the process of NET formation.

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### 5.4. Reorganization of membrane structures-the role of autophagy in NETosis

While the decondensated nuclear content expands, the space between the two membranes of the delobulated nuclear envelope starts growing, this which eventually leads to formation of vesicles and disintegration of nuclear membranes. During the final stage, nuclear and granular integrity is completely lost, which allows mixing of the chromatin and the granular components, and a rupture in the plasma membrane causes the release of extracellular chromatin traps.

However, vesicle formation is also seen in neutrophils isolated from CGD patients, which are unable to produce NETs [73]. This observation suggests that vesicles do not necessarily originate from the nuclear envelope, but ER membranes are likely to be assembled as a source of autophagic vesicles [34], in addition to possible *de novo* vesicle formation. A decrease in perinuclear ER membranes may result in lower morphological constraints on nuclear collapse, and calcium leaking form the ER may activate PAD4. Taken together, these events could partially explain that autophagy is needed for nuclear decondensation and NET formation [73]. These speculations are supported by the finding that inhibition of mTOR, a suppressor of autophagy, also leads to enhanced NET production (see before [10]).

## 6. NETs and haemostasis

NETs are a newly recognized scaffold of venous [74] and arterial [75,76] (Figure 3.) thrombi (besides fibrin and von Willebrand Factor [vWF]) that allows cell localization (neutrophils, red blood cells), platelet activation and aggregation, and promotion of both (extrinsic and intrinsic) pathways of coagulation. Thus, NETs are a focus of cross-talk between immunity, inflammation and haemostasis. Here we discuss the interaction among the various players of the haemostatic system and NET components.

### 6.1. NETs and the vessel wall

The classic view of the intact endothelial surface emphasizes its anticoagulant role. While endothelial damage is a common initiator of arterial thrombosis, in the case of deep vein thrombosis (DVT), activation of endothelium and Weibel-Palade Body (WPB) release play a crucial role. NETs induce endothelial cell damage and death [17,77-79], an effect that is likely to be assigned to NET-associated proteases, defensins and, most importantly, histones [78,80]. Binding of histones to membrane phospholipids results in pore formation and influx of ions [81-83], this may lead to elevated endothelial calcium levels, vWF release from WPB [84], activation of endothelium, or even endothelial cell death. Endothelial ROS formed under these circumstances may, in turn, trigger NET formation by neutrophils [77]. Perfusion of iliac artery cross sections with NE results in increased thrombogenicity of the arterial wall [85], although it is not clear if NET-bound NE is able to reproduce this effect at the site of vascular damage.

NETs also contribute to the progression of atherosclerotic plaque formation in the subendothelial layer of arteries: neutrophils infiltrate arteries during early stages of atherosclerosis [86], and NETs can be detected in murine and human atherosclerotic lesions [87].

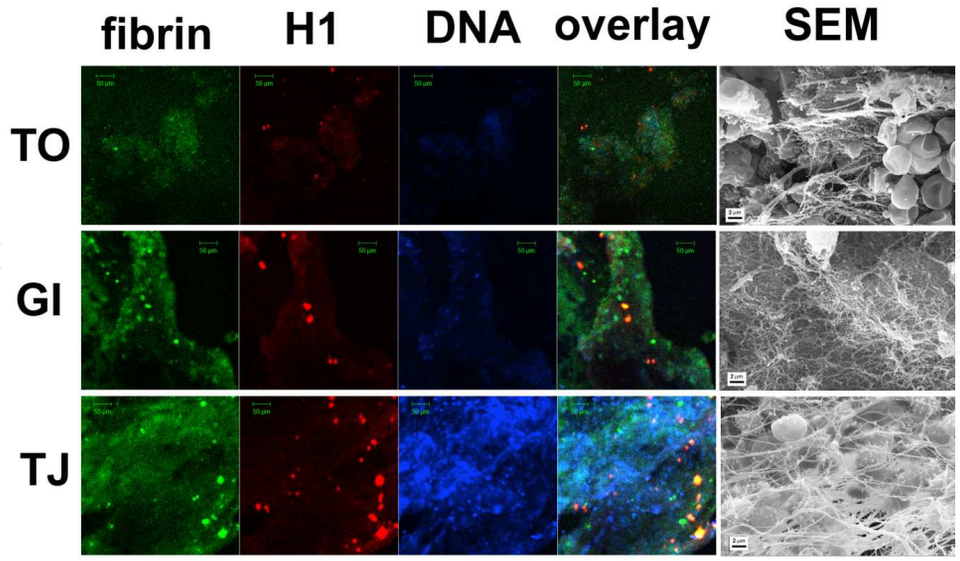


**Figure 3.** Presence of NET components in arterial thrombi. Following thrombectomy thrombus samples were either frozen for immunostaining or washed, fixed and dehydrated for SEM processing. Sections of frozen samples were double-immunostained for fibrin (green) and histone 1 (red) as well as with a DNA-dye, TOTO-3 (blue). Images were taken at original magnification of ×20 with confocal laser microscope. SEM images were taken from the fixed samples of the same thrombi. There was variable but widespread staining for DNA. Histones were also present though were not so widely dispersed and in some cases were coincident with fibrin aggregates. The size of the thrombus-section area staining for DNA and histone correlated with the leukocyte content of the respective thrombus observed in the SEM images. The red blood cell-rich (TO) and the fibrin-rich (GI) thrombi showed limited DNA-and histone-positive regions in contrast to the extensively stained areas in the leukocyte-rich (TJ) thrombus. TO: a thrombus from popliteal artery, GI: a thrombus from infrarenal aorta aneurysm, TJ: a thrombus from femoro-popliteal graft. Figure from [76].

### 6.2. NETs and platelets

NET fibres bind platelets directly and/or indirectly, and support their aggregation [88]. When perfused with blood, NETs bind platelets serving as an alternative scaffold for platelet adhesion and activation [89].

The first step of platelet binding involves either electrostatic interactions between NET histones and platelet surface phospholipids [81]/carbohydrates [90], or histone binding to Toll-like receptors 2 and 4 [91]. Platelets also bind double and single stranded DNA in vitro [92,93]. Adhesion molecules may also play a role in thrombocyte-NET interactions, such as vWF (binding histones through its A1 domain) [94], fibronectin or fibrinogen [89,84]. The interaction of histones with platelets results in calcium influx either by pore formation [95] or by opening of existing channels [96], a process, which triggers activation of $\alpha$IIb$\beta$3 [97]. This chain of events raises the possibility of a sequential histone-induced activation of platelets (first binding to platelet surface, then, following activation, binding to adhesion molecules [88]), which could explain the unsaturable nature of histones binding to platelets [88]. When infused into mice, histones co-localize with platelets and induce thrombocytopenia and thrombosis [83,84,88], possibly partially through potentiation of thrombin-dependent platelet-activation [98].

Serine proteases may also play a role in platelet activation: NETs contain enzymatically active neutrophil elastase NE and cathepsin G [23], and these proteases potentiate platelet aggregation through proteolitically activating platelet receptors [99,100]. Some of these elements, however, play an ambiguous role in the modulation of platelet functions: e.g. NE is also an effective enzyme for the cleavage of vWF under high shear stress [101], helping the detachment of platelets from thrombogenic surfaces.

NETs also seem to bind certain interleukins that may enhance platelet activation and aggregation: the presence of IL17A and-F was shown in NET regions of acute myocardial infarction thrombus specimens [102].

Platelet-NET interaction seems to be bidirectional in many ways. Serotonin released from platelets promotes the recruitment of neutrophils [103]. Activated platelets generate ROS, such as superoxide [104], and secrete human β-defensin 1 [105], both of which can trigger formation of NETs [13,106]. Platelets pre-stimulated with LPS or collagen also induce NETosis in neutrophils [17,108], contributing to the formation of a vicious cycle of NET formation and platelet activation [74].

Interaction between platelets and NETs might also be involved in pathological situations like transfusion-related acute lung injury (TRALI) [108,109], thrombotic microangiopathies [110], or heparin-induced thrombocytopenia (HIT). During HIT, possible binding of NETs to PF4 forming an antigenic complex may offer an explanation for disease progression even after immediate removal of heparin [111].

### 6.3. NETs and red blood cells

Red blood cells are no longer considered as passively entrapped elements of thrombi, but cells that may promote thrombosis by exposing phosphatydilserine and altering blood viscosity [112]; furthermore, their presence modulates structural parameters of the forming fibrin meshwork through integrin-mediated fibrin(ogen)-red blood cell interactions [113].

Similarly to platelets, RBCs avidly bind to NETs after perfusion of whole blood [89], possibly through direct and indirect mechanisms. RBCs can bind DNA, since it was eluted from the surface of isolated RBCs from cancer patients [114]. Activated neutrophils or platelets (e.g. in NETs) can also recruit RBCs at very low venous shear in vitro [115]. NETs are predominantly found in the red, RBC-rich part of experimental mice DVT thrombus, suggesting that NETs could be important for RBC recruitment to venous thrombi [84].

### 6.4. NETs and the coagulation system

NETs offer a variety of activators for both the extrinsic and the intrinsic (contact-) pathways of the coagulation cascade [116,107] stimulating fibrin formation and deposition in vitro [89,107,116] (Figure 4.).

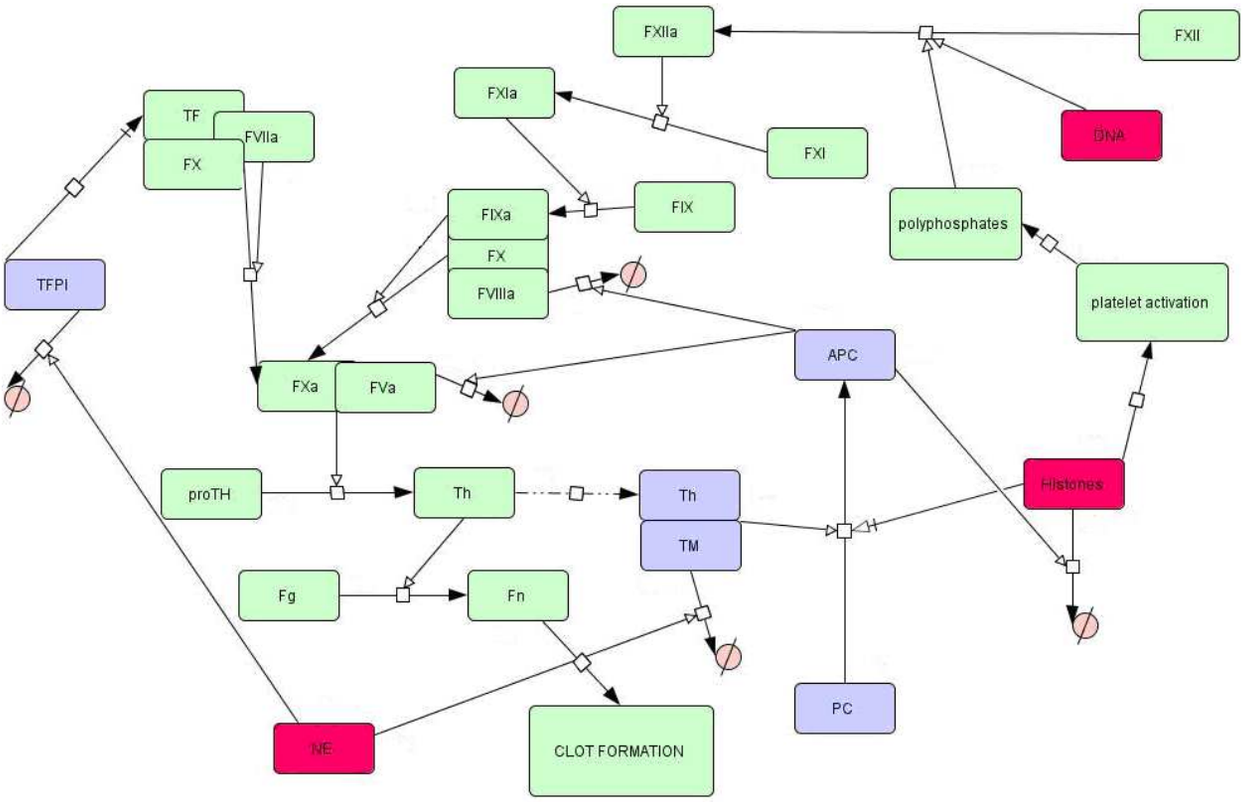


**Figure 4.** Examples of NET-coagulation interactions. Green boxes indicate prothrombotic elements/steps of the cascade. Blue represents antithrombotic systems. Red boxes stand for NET components. Dashed pink circles symbolize degradation of the respective protein. Dashed arrows represent inhibition, while arrows pointing to the middle of another arrow represent activation of a process. For further explanation, see text.

NE and cathepsin G, two serine proteases that are in the NETs, degrade inhibitors of coagulation [12]. NE is known to cleave tissue factor pathway inhibitor (TFPI) of the *extrinsic pathway*, and enhance factor Xa activity [117]. The cleavage of TFPI by NE is supported by activated platelets that attach to the surface of neutrophils and facilitate NET formation [107]. Neutrophil-expelled nucleosomes also bind TFPI and serve as a platform for the NE-driven degradation of TFPI [107]. NETs do not only release brakes of the extrinsic pathway, but also trigger it: TF was identified as a NET component [116,118]; and disulphide isomerase (PDI) released from damaged or activated endothelial cells and platelets (e.g. in NETs) participates in bringing the inactive (encrypted) TF (e.g. in neutrophils [75,119] and platelets [120,121]) to its active (decrypted) form [122].

NETs also bind factor XII and stimulate fibrin formation via the *intrinsic coagulation pathway* [116]. Faxtor XII can be activated following contact with pathogens (e.g. entrapped in NETs), damaged cells (e.g. endothelial damage by NETs), and negatively charged surfaces (such as the NET component DNA, which also enhances the activity of certain coagulation serine proteases [123]). Polyphosphates released from activated platelets following stimulation by histones may also serve as coagulation-triggering negatively charged molecules [91,124].

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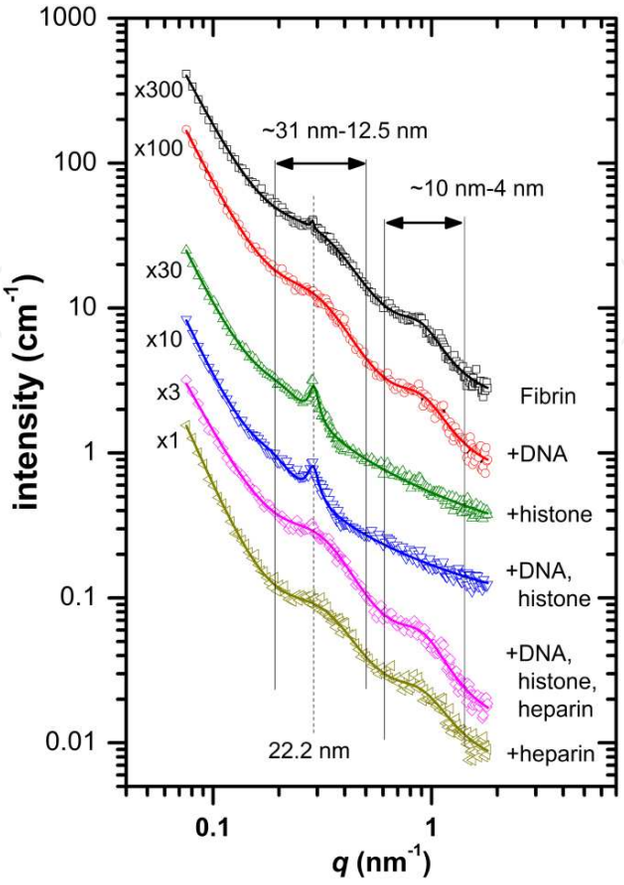


**Figure 5.** Small-angle X-ray scattering in fibrin clots containing DNA, histone, heparin or their combinations at the same concentrations. The general decay trend of the scattering curves reflects the fractal structure of the fibrin clot and its effect can be modeled as a background signal with empirical power-law equations. The peaks arising above this background reflect the longitudinal and cross-sectional alignment of fibrin monomers. A small, but sharp peak in pure fibrin at q-value of ≈0.285 nm-1 corresponds to the longitudinal periodicity of d=2π/q'=22 nm that is in agreement with earlier SAXS studies [128] and a little bit lower than the values reported for dried samples in transmission electron microscopic investigations [129]. This peak cannot be resolved in fibrin containing DNA or heparin indicating that these additives disrupt the regular longitudinal alignment of the monomeric building blocks. In contrast, the addition of histone does not interfere with the longitudinal periodicity, the related scattering peak is even more pronounced. In pure fibrin two additional broad scattering peaks can be resolved spanning over the q-ranges of ≈0.2 to 0.5 nm-1 and ≈0.6 to 1.5 nm-1. The first peak can be attributed to periodicity of ≈12.5 to 31 nm in cluster units of the fibers, while the second one corresponds to periodicity of ≈4 to 10 nm characteristic for the average protofibril-to-protofibril distance based on the structural models of Yang et al. [130] and Weisel [129]. Both of these broad peaks are most profoundly affected by the presence of histone (a 10-fold decrease in the area of Peak 1 and complete loss of Peak 3) suggesting that this additive interferes with the lateral organization of protofibrils resulting in lower protofibril density. Earlier studies [131] have shown that lower protofibril density can correspond to thicker fiber diameter, which is in qualitative agreement with SEM results [76]. The structure modifying effects of histone are preserved in the presence of DNA, but these effects are completely reversed in the quaternary system of fibrin/DNA/histone/heparin; Curves are shifted vertically by the factors indicated at their origin for better visualization. Symbols represent the measured intensity values, while solid lines show the fitted empirical functions. The dashed vertical line indicates the longitudinal periodicity of fibrin of about 22 nm (representing the approximate half-length of a fibrin monomer), while the solid vertical lines show the boundaries of the broad peaks that characterize the lateral structure of the fibrin fibers. q (momentum transfer)=4π/λ sinθ, where θ is half the scattering angle and λ is the wavelength of the incident X-ray beam. Figure from [76].

Besides its crucial role in NET-driven thrombosis [125], PAD4 has also been shown to citrullinate antithrombin (ATIII) in vitro [126], which weakens its thrombin-inhibiting efficiency and this may be an additional factor contributing to increased thrombin generation associated with NETs. Histones also bind to fibrinogen and prothrombin [127], and can aggregate vWF [94], the significance of which is not clear.

NET components also interfere with the *anticoagulant* systems in plasma. Despite the historically attributed anticoagulant properties of histones [131,132] (prolonging the plasma based standard clotting assays, probably due to their affinity for negatively charged phospholipids, such as phosphatidylserine [81]), nowadays they are viewed as clear procoagulant substances, due to their platelet-activating nature (see before) and their modulatory effects on the thrombin-thrombomodulin(TM)-activated protein C (APC) pathway. Histones interact with *TM* and *protein C* and inhibit TM-mediated protein C activation [134]. Interestingly, in return, APC cleaves histones (H2A, H3, H4) and reduces their cytotoxicity [83], possibly serving as a basis for a counter-regulatory process. Cleavage of histones is relatively slow, but is augmented substantially by membrane surfaces, particularly those that best support APC anticoagulant activity [83], although NET-bound histones may be more difficult to cleave [78]. Thrombomodulin is also cleaved by NE and may also be rendered inactive by neutrophil oxidases (such as MPO) [135,136] present in NETs.

*Heparin*, a highly sulfated polyanion (GAG) is able to interfere with DNA-histone complexes [76] (Figure 5.). Heparin can remove histones from NET chromatin fibres, leading to their destabilization [89,116]: NETs are dismantled after perfusion with heparinized blood [116]. Heparin also blocks the interaction between the positively charged histones and platelets [74], in this way adding newly recognized elements to its long-known anticoagulant effects.

### 6.5. NETs, thrombolysis, NET lysis

Whilst there are extensive studies on the interaction between NET components and coagulation, little is known about their effects on fibrinolysis.

Histones and DNA, representing the main mass of NETs, seem to have *antifibrinolytic* properties. Addition of purified histones and DNA to the forming clot in vitro results in altered clot structure seen under SEM, a finding also confirmed by short axis X-ray scattering (SAXS) [76] (Figure 5.). The structural changes are accompanied by increased mechanical (Figure 6.) and enzymatic resistance of the clot, and a change in the microscopic properties of the lysis front (Figure 7.), especially when DNA and histones are used in combination [137,76]. Lysis may be also delayed by NET components resulting from interactions between fibrin degradation products (FDPs) and DNA/histones [76].

Nevertheless, certain NET components may *promote* thrombolysis: in vitro studies have shown that NE and cathepsin G can degrade fibrin [138], and in plasminogen-knockout mice, more neutrophils infiltrate the clot [139], possibly serving as an auxiliary mechanism when plasmin-mediated fibrinolysis is impaired [140]. Histone 2B can serve as a receptor to recruit plasminogen on the surface of human monocytes/macrophages [141], and perhaps in NETs as well, where the co-localization of NE and plasmin(ogen) could result in amplified formation of mini-

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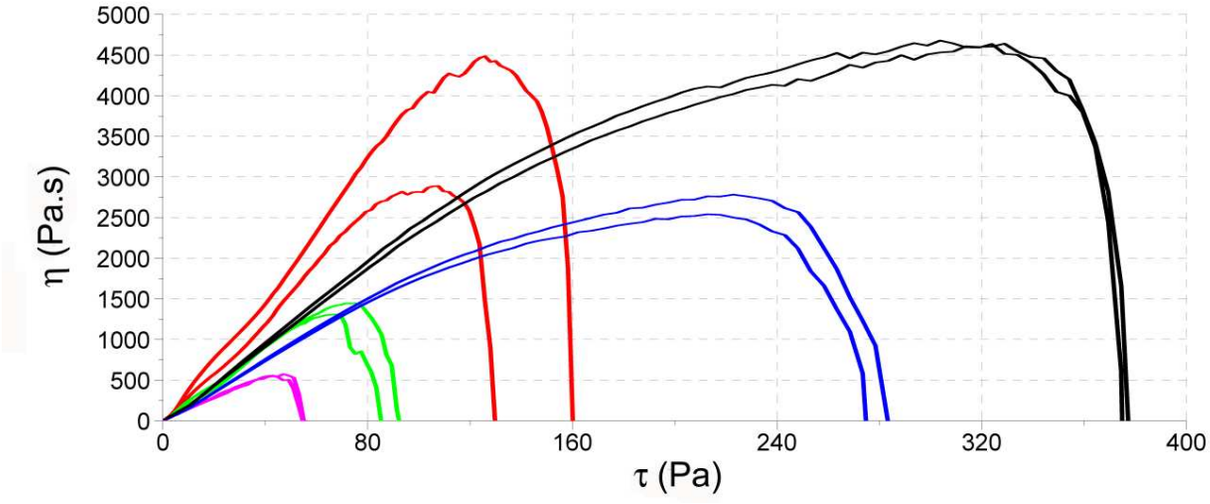


**Figure 6.** Rheometer studies showing the effect of DNA and histones on the critical shear force needed to disassemble fibrin. Curves are shown for pure fibrin (red), fibrin containing increasing DNA concentrations (green<magenta), histone (blue) and histone+DNA (black). In the presence of DNA alone the curves can be interpreted as increased sensitivity of fibrin to mechanical shear so that the shear force needed to disassemble fibrin (where viscosity approaches zero) is reduced in comparison to the situation without DNA. However, when histones are added to fibrin, and to a greater extent when histones are added to fibrin+DNA, the clots become more stable and resistant to shear forces. τ: shear stress, η: viscosity. Figure from [76].

plasmin, a plasmin-derivative that bears a catalytic efficiency on cross-linked fibrin that exceeds that of plasmin [142]. NE is also able to efficiently disable the major plasmin-inhibitor, $\alpha$2-antiplasmin, further supporting plasmin action. PAD4 is eventually secreted from neutrophils during NET formation and was shown to citrullinate fibrin in rheumatoid arthritis [144] (although less efficiently than PAD2 [145]), but the significance of this related to thrombolysis is not known.

In vitro and in vivo observations indicate that fibrin, vWF and chromatin form a co-localized network within the thrombus that is similar to extracellular matrix [84,82,116], and it is likely that each of these components should be cleaved by their own appropriate enzyme (plasmin, ADAMTS-13, and DNAses), therefore it is important to assess current knowledge on the possible ways of NET degradation in blood plasma.

NETs can be degraded by *DNases* in vitro. There are two main DNases in human plasma: DNase1 and DNase1-like family, out of which, DNase1-like 3 (DNase1l3) is the most characterized. Both enzymes show calcium/magnesium dependency. DNase1 is secreted into circulation by a variety of exocrine and endocrine organs [146-148], whereas DNase1l3 is released from liver cells, splenocytes, macrophages and kidney cells [149]. DNase1 and DNase1l3 cooperate during in vitro chromatin breakdown (chromatin fragmentation is completely absent if DNase1 and DNase1l3 is inhibited) [150], and preprocessing of NETs by DNAse1 also facilitates their clearance by macrophages [151]. Plasmin is able to cleave histones [152], thus helping DNase action, since DNase1 prefers protein-free DNA. In addition, NE already present in NETs, APC (see before), thrombin [153] and an unidentified protease [154]

may also assist in histone degradation. The in vivo relevance of plasmin-DNase cooperation is reflected in the elevated levels of plasma DNA in patients with DVT [74].

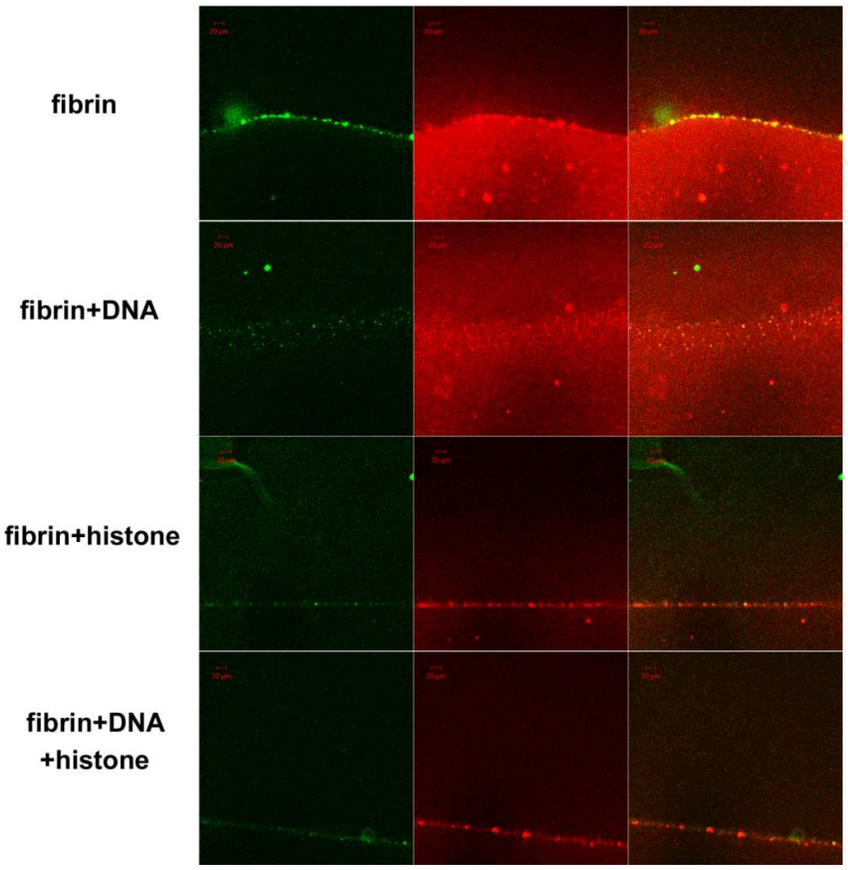


**Figure 7.** Confocal microscopy studies of lysis front movement using green fluorescent protein-labeled tPA (tPA-GFP) and red fluorescent fibrin after 25 min of fibrinolysis. Each column of micrographs from left to right shows green tPA-GFP fluorescence, red AlexaFluor 546 conjugated fibrin fluorescence and the merged image. The first row shows the accumulation of fibrin aggregates that co-localize with tPA-GFP. The second row, with the addition of DNA, shows less fibrin aggregate formation but a diffuse fibrin clot that remains behind the advancing tPA-GFP front. The lower two rows where clots contain histones and histones+DNA, respectively demonstrate reduced formation of fibrin aggregates within fibrin and less binding of tPA-GFP. Figure from [76].

As a possible counter-regulatory mechanism, NETs seem to protect themselves from bacterial and perhaps human DNases by limiting the availability of divalent cations (see calprotectin) and consequently the activity of these enzymes [155].

## 7. Conclusion

NETs are 'double-edged swords' of innate immunity. While they seem to be protective against a wide range of pathogens, their contribution to various diseases, and their clear prothrombotic role in the circulation may have dangerous consequences to the host. In terms of thrombosis, they seem to serve as a fundamental scaffold that supports thrombus integrity by providing

a surface for activation of procoagulant proteins and platelets, in both venous and arterial thrombi. Further investigation is indispensable to determine their exact role in the process of thrombi dissolution, and to test whether breakdown of NETs (e.g. by DNases) increases the therapeutic efficiency of the current thrombolysis protocols.

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